Current Biology 19, 1821–1826, November 17, 2009 ©2009 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2009.09.023

Report

Oriented Cell Division as a Response to Cell Death and Cell Competition

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Summary

The imaginal disc epithelia that give rise to the adult ectoderm of *Drosophila* can compensate to produce normal adult organs after damage. We looked at the local response to cell death by using two genetic methods to elevate cell death rates. During cell competition, sporadic cell death occurs predictably along the boundaries between populations of competing wild-type and "Minute" cells (*M*/+) [1]. Boundaries between *M*/+ and wild-type populations show an unusual degree of mixing, associated with mitotic reorientation of wild-type cells toward *M*/+ territory that they take over. Apoptosis of *M*/+ cells was the cue, and reoriented mitosis required the planar cell polarity genes *dachsous*, *fat*, and *atrophin*. Clones mutated for *pineapple eye*, an essential gene, elevate apoptosis by a noncompetitive mechanism [2]. Mitosis was also reoriented near cells mutant for *pineapple eye*, likewise dependent on the planar cell polarity genes. These findings show that planar cell polarity genes are required for responses to cell death. Oriented mitosis may help maintain morphology as dividing cells replace those that have been lost.

Results and Discussion

Cell Death Reorients Mitotic Figures

Many organisms can regulate their development in response to cell death or damage. Concentrated damage stimulates wound healing, but responses to the sporadic death of single cells are poorly understood. During cell competition, sporadic cell death occurs predictably along the boundaries between populations of competing genotypes [1]. The boundaries between competing genotypes also acquire unusually irregular courses, the basis for which has not been investigated. Cell migration is normally minimal in imaginal discs, and clones of cells derived from any marked precursor generally grow as coherent patches [3].

Unusual boundaries were first noticed when mitotic recombination was used to create clones of "Minute" cells (*M*/+), heterozygous for any one of many ribosomal protein genes [4], in predominantly +/+ compartments. Although *M*/+ flies are viable, *M*/+ clones in a wild-type compartment exhibit high rates of cell death and are eliminated by this cell competition [5, 6]. Prior to their elimination, *M*/+ clones are dispersed into small patches surrounded by +/+ cells [5]. Conversely, even a small number of +/+ cells can progressively take over an *M*/+ developmental compartment [7]. In our experiments, the boundaries of +/+ clones were unusually jagged and irregular, indicating an unusual mixing between the cell populations as +/+ cells infiltrate formerly *M*/+ territory (Figures 1A–1G).

The antiapoptotic baculovirus protein p35 was expressed to investigate whether cell death contributed to the irregular boundaries. Baculovirus p35 reduced the degree of intermingling between +/+ and *M*/+ cell populations (Figures 1H and 1I). Therefore, intermingling of +/+ cells with the remaining *M*/+ cells appears to be a response to death of individual *M*/+ cells.

It was surprising that cell death would create irregular boundaries between competing genotypes, because *M*/+ cells mostly surrounded by +/+ cells tend to die [1], which should smooth the boundaries by removing the most intermingled cells. We found that the number of mitotic figures was little affected by cell competition (Figure 2A) but that the plane of mitosis was reoriented near competing boundaries. Because regional biases in mitotic orientation occur during normal development [8], mitotic orientation was measured with respect to boundaries between +/+ clones and *M*/+ cells rather than to global coordinates (Figure 2B). Because clone boundaries take many orientations, divisions of wild-type cells showed almost random orientation with respect to nearby clone boundaries, regardless of location in the disc (Figure 2C). By contrast, during cell competition, the divisions of +/+ cells tended to be oriented perpendicular to nearby clone boundaries, directed toward the nearest *M*/+ cells (Figure 2D). Oriented cell division might be the cause of intermingling at boundaries between competing populations, and some examples were seen where dividing +/+ cells appeared to encroach on the *M*/+ territory (Figure 2E; see also Figure S1 available online). The mitotic orientation of *M*/+ cells on the other side of the boundary was much less affected (Figure 2F). Orientation of +/+ cells was lost when death of *M*/+ cells was prevented by coexpression of baculovirus p35 and dominant-negative Dronc (Figures 2G and 2H). Dominant-negative Dronc inhibits certain p35-independent aspects of apoptosis [9]. Therefore, death of *M*/+ cells during cell competition orients the division of nearby +/+ cells to direct newborn +/+ cells into previously *M*/+ territory.

To test whether oriented cell division was specific for cell death caused by cell competition, we examined clones of cells mutant for the *pineapple eye* (*pie*) gene. The *pie* gene encodes a PHD-finger protein required for normal cell viability, so that clones of *pie* homozygous cells contain many apoptotic cells while enough cells survive that *pie* homozygous clones are large enough for study (Figure 2I) [2]. The apoptosis is not caused by cell competition because it does not require nearby +/+ cells. Mitotic +/+ cells were oriented by nearby clones of *pie* homozygous cells, showing that oriented cell division is not specific for competitive cell death but is a more general response to nearby apoptosis (Figure 2J). We also noted that homozygous *pie* mutant clones had highly irregular outlines, like those of competing cell populations (Figure 2I).

Planar Cell Polarity Genes that Orient Mitosis

A subset of the genes that are also important for planar cell polarity (PCP) have recently been found to play a role in mitotic

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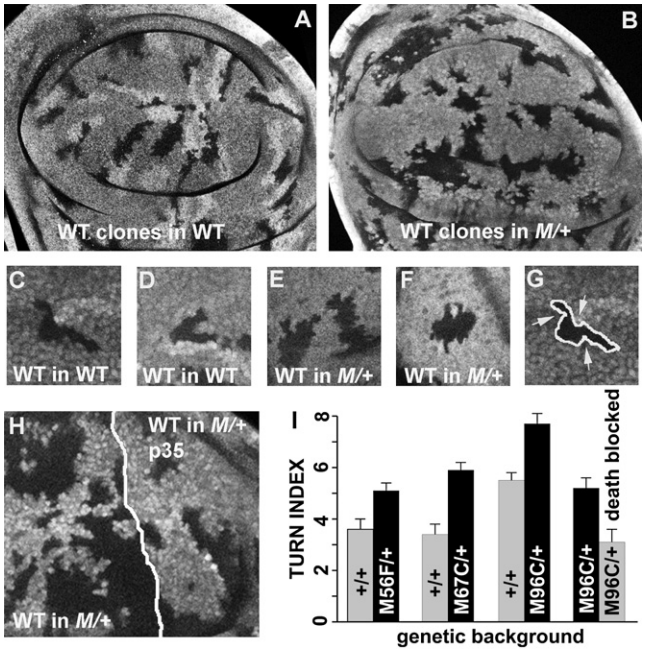

Figure 1. Intermingling between Wild-Type and *M*/+ Populations

(A) A wing imaginal disc dissected 72 hr after clone induction (see Experimental Procedures) showing clones of wild-type (WT) cells lacking staining for β-galactosidase and their sister clones with twice the β-galactosidase transgene dose. Note the tendency for clones to grow away from the center of the wing disc, the future distal part of the adult wing.

(B) Clones of wild-type cells dissected 48 hr after clone induction (see Experimental Procedures) lacking staining for β-galactosidase in an *M*/+ wing. The *M*/*M* sister clones do not survive. The boundaries between cell populations are noticeably more irregular than in (A), and many small "islands" of *M*/+ cells have been split off.

(C–F) Examples of similarly sized clones of wild-type cells in a wild-type disc (C and D) or in an *M*/+ background (E and F), illustrating that clone boundaries are less smooth in the *M*/+ background.

(G) A "turn index" is determined from the tracing of the clone boundary. See Experimental Procedures for details.

(H) Wild-type clones, lacking β-galactosidase, in the *M(3)96C*/+ background. Baculovirus p35 expression is driven in the posterior compartment to prevent cell death. The posterior compartment boundary (white line) was identified by labeling for Ci155 protein expression (not shown). Expression of p35 leads to smoother clone boundaries in the posterior compartment.

(I) Quantification of turn index. Wild-type clones (FRT42, FRT80, and FRT82) have smoother boundaries in wild-type than in *M*/+ backgrounds. Blocking cell death in the *M(3)96C*/+ background by expression of baculovirus p35 also produces smoother boundaries. Cells were dissected 72 hr after clone induction (see Experimental Procedures). Error bars represent standard error of the mean (SEM).

orientation during normal development and to contribute to the normal morphology of the wing. Mitotic figures tend to orient toward the dorsoventral boundary in much of the wing portion of the wing imaginal disc, and cell clones are typically longer in the proximodistal axis, prefiguring the elongated proximodistal axis of the wing itself [8] (Figures 3A–3C). This mitotic orientation is randomized in mutants of *dachsous*, a PCP gene that encodes an atypical cadherin also required as a tumor suppressor. Accordingly, *dachsous* mutant flies have a rounded wing shape. Mitotic orientation is also affected by overexpression of Fat, another atypical cadherin tumor suppressor required for PCP that is a heterophilic binding partner of Dachsous [8, 10–12]. We examined *fat* mutations to confirm through loss-of-function studies that *fat* was required for mitotic orientation (Figures 3C and 3D). In contrast to in wild-type cells, mitotic figures in clones of cells homozygous for a *fat* null mutation were unoriented with respect to the dorsoventral boundary. Such clones were round, and their boundaries were smooth (Figure 4Q and data not shown). Thus, *fat* is also required for mitotic orientation in the normal wing, along with *dachsous*.

The Fat intracellular domain interacts directly with the Atrophin protein, a transcriptional repressor that functions within the nucleus and is a homolog of Atrophin 1, in which expansion of a CAG domain is associated with human dentatorubral-pallidoluysian atrophy, an autosomal-dominant neurodegenerative disease [13–16]. Mutations in *atrophin* (*atro*) are embryonic lethal, but clones of null mutant cells exhibit planar polarity defects and grow with smooth boundaries, similar to *dachsous* or *fat* mutants [16]. We examined mitotic figures in *atro* mutant clones to determine whether *atro* was also required for mitotic orientation. We found that *atro* mutant cells showed no particular orientation with respect to the proximodistal axis (Figures 3G and 3H). The *atro* mutant clones also had smooth boundaries (Figures 3E and 3F). Thus, the *atrophin* gene appears to function in the same pathway as *fat* and *dachsous* in orienting mitosis in normal development.

Smooth interfaces between cell populations can indicate affinity differences between cells [17]. To test the effect of cell adhesion, we examined clones of cells that overexpressed E-cadherin. These have smooth boundaries, like *fat*, *dachsous*, or *atro* mutant clones [17]. The orientation of mitosis with respect to the proximodistal axis was not affected by overexpression of E-cadherin, however (Figures 3I and 3J). We suggest that, whereas cells overexpressing E-cadherin form smooth boundaries as a result of cell affinity differences [17], *fat*, *dachsous*, or *atro* mutations do so through random mitotic orientation, their clones growing like colonies in the tissue. We cannot exclude that cell adhesion differences might also contribute, given heterophilic binding between Fat and Dachsous [18] and given that Atro might affect transcription of cell adhesion molecules.

Planar Cell Polarity Genes Are Required in Response to Cell Death

To determine whether mitotic orientation in response to cell death also depends on the same PCP genes, clones of *dachsous* (*ds*), *fat* (*ft*), or *atro* homozygous cells were examined in *M*/+ backgrounds, and their mitotic orientations were measured with respect to the clone boundaries. Unlike clones of +/+ cells, mitosis of *ds* mutant, *ft* mutant, or *atro* mutant cells was not oriented by the boundary with *M*/+ cells (Figures 4A–4F). The boundaries of the mutant clones remained smooth in the *M*/+ background (Figures 4G–4J and data not shown). Thus, these PCP genes are required to reorient cell division in response to competitive cell death, as well as for oriented cell division in normal development.

The failure of *dachsous*, *fat*, and *atro* mutant cells to orient in response to *M*/+ cells could be explained in a trivial way if they did not compete with *M*/+ cells, because we have already noted that mitotic orientation depends on death of *M*/+ cells (Figures 2G and 2H). This was plausible because competitive death of *M*/+ cells is increased by the proximity and degree of their exposure to non-Minute cells [1]; such exposure may be reduced by the smooth boundaries with *fat* and *atro* clones. Indeed, compartment boundaries, across which no intermingling occurs, are also barriers to competition [5, 19, 20].

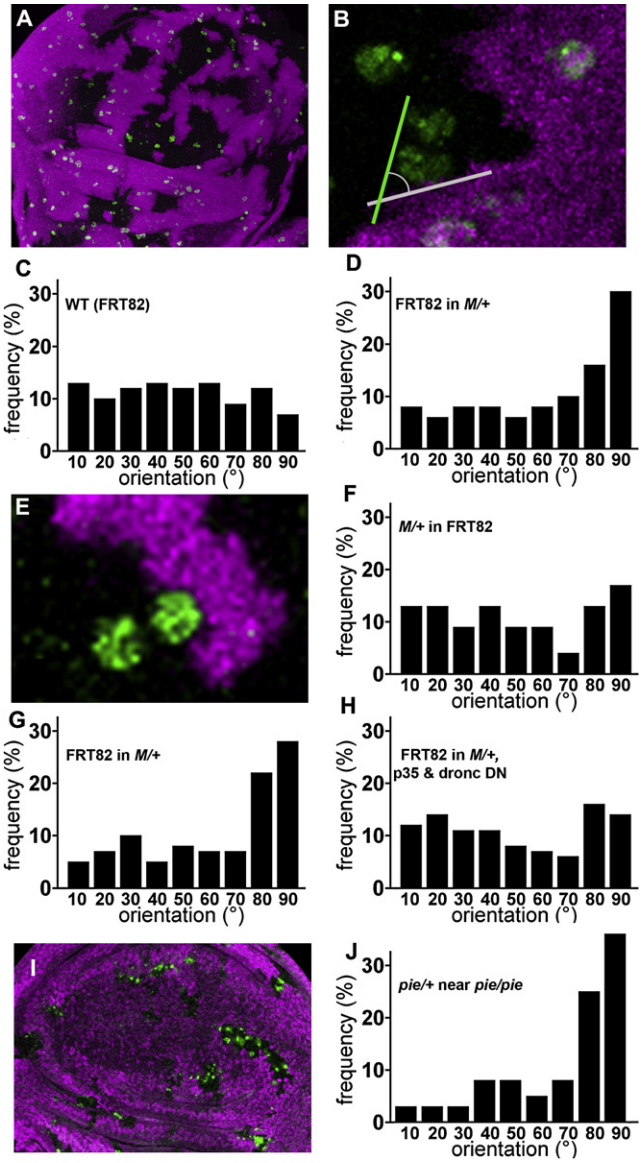

Figure 2. Cell Death Orients Mitosis

(A) Wing pouch region of an imaginal disc containing wild-type clones that are unlabelled for β-galactosidase in the magenta *M*/+ background. Mitotic figures have been labeled for phosphohistone H3 in green and are distributed all across the tissue.

(B) Wild-type anaphase oriented approximately 70° to the boundary with nearby *M*/+ cells.

(C) Mitotic figures in wild-type clones (FRT82) show little orientation with respect to their clone boundaries. n = 75.

(D) *M(3)96C*/+ cells strongly orient mitotic figures in wild-type clones. Nearly 50% of mitotic figures are oriented >70° to the boundary, compared to less than 20% in the control in (C). n = 71.

(E) An example of a mitotic wild-type cell apparently deforming the boundary with nearby *M*/+ cells. The same confocal data are shown reconstructed from the side in Figure S1.

(F) Mitotic figures of *M(3)96C*/+ cells show little orientation toward wild-type clones. n = 50.

(G and H) Orientation of wild-type mitoses in the *M(3)96C*/+ background (G) is suppressed when cell death is prevented by expression of baculovirus p35 and dominant-negative Dronc (H). n = 164 in (G); n = 122 in (H).

(I) Clones of *pie* mutant cells (lacking β-galactosidase staining in magenta) exhibit high levels of apoptosis (anti-activated caspase labeling in green). The turn index for these *pie* clones was 11.01 ± 0.88, compared with 7.28 ± 0.86 for parallel FRT40 control clones.

Potential effects of *dachsous* or *fat* mutations on cell competition were hard to assess, because these mutant cells are themselves hyperplastic [10]. The *atro* mutations were not hyperplastic, however. Clones of cells mutant for *atro* grew at a rate similar to controls and did not cause cell death (Figure 3E; Figure 4K; data not shown). This permitted measurement of the effect of *atro* mutations on cell competition. If *atro* mutant cells did not compete with *M*/+ cells, we would expect *atro*/*atro* clones to grow less in an *M*/+ background than wild-type cells do, and not to kill neighboring *M*/+ cells as wild-type cells do. We found, however, that *atro*/+, *M*/+ cells were outcompeted by *atro*/*atro* clones almost as well as by wild-type clones, despite the smooth boundaries between them (Figures 4G–4J). Clones of *atro* mutant cells grew rapidly in an *M*/+ background, occupying large proportions of the tissue, like clones of +/+ cells. The *atro*/*atro* clones were slightly smaller in the Minute background, but the difference was not statistically significant (Figures 4K and 4L). Like *M*/+ cells next to +/+ cells, *atro*/+, *M*/+ cells died next to *atro*/*atro* cells (data not shown). The rate of death was reduced in absolute terms compared to *M*/+ cells next to +/+ cells but similar when adjusted for the shorter boundaries of the smooth *atro* clones (Figures 4M and 4N). These experiments confirmed that *atro*/*atro* cells compete with and kill *M*/+ cells, and therefore that *atro* is required for reorienting cell division in response to *M*/+ cell death. We could not assess the consequences of failing to reorient cell divisions for wing shape because of the additional roles of *atro* in adult wing differentiation [21]. We also found that *M*/+ cells continued to die next to clones of +/+ cells given smooth boundaries through overexpression of E-cadherin. There was death within E-cadherin overexpression clones as well (data not shown), presumably because of dominant-negative effects of E-cadherin overexpression on Wingless survival signaling [22].

We also assessed whether PCP genes were required to reorient mitosis in response to the noncompetitive death of *pie*/*pie* cells. Mitotic recombination was induced in the *ft* +/+ *pie* genotype to obtain *ft*/*ft* clones next to *pie*/*pie* clones. The *ft*/*ft* cells adjacent to *pie*/*pie* clones showed random mitotic orientation, whereas the *ft* +/+ *pie* cells that were near to *pie*/*pie* clones reoriented (Figures 4O and 4P). Whereas the boundaries between *pie*/*pie* clones and *ft* +/+ *pie* cells were irregular, boundaries between *pie*/*pie* clones and *ft*/*ft* clones were smooth, as were boundaries between *ft*/*ft* clones and *ft* +/+ *pie* cells (Figure 4Q). Because cell death still occurred in *pie*/*pie* clones near to *ft*/*ft* cells (data not shown), these mitotic orientation data provide further evidence that PCP genes are required to reorient mitosis in response to cell death.

Because competitive cell death is compensated by growth [1] and because Fat can affect the Hippo-Salvador-Warts (HSW) pathway of tumor suppressors [23], we wondered whether HSW activities might be altered where +/+ and *M*/+ cells abut. However, we were unable to detect changes in the expression of the Expanded or Merlin proteins or the *expanded* LacZ enhancer trap, or in the nuclear localization of the Yorkie protein where +/+ and *M*/+ cells are adjacent (data not shown); these are all reporters of HSW activity [23].

Conclusions

Our main conclusion is that cell death orients nearby cell division in wing disc development. We suggest that regulative

(J) Within regions of wild-type cells (*pie[E1-16] FRT40*/+), mitoses tend to orient toward nearby boundaries with *pie* homozygous clones. n = 106.

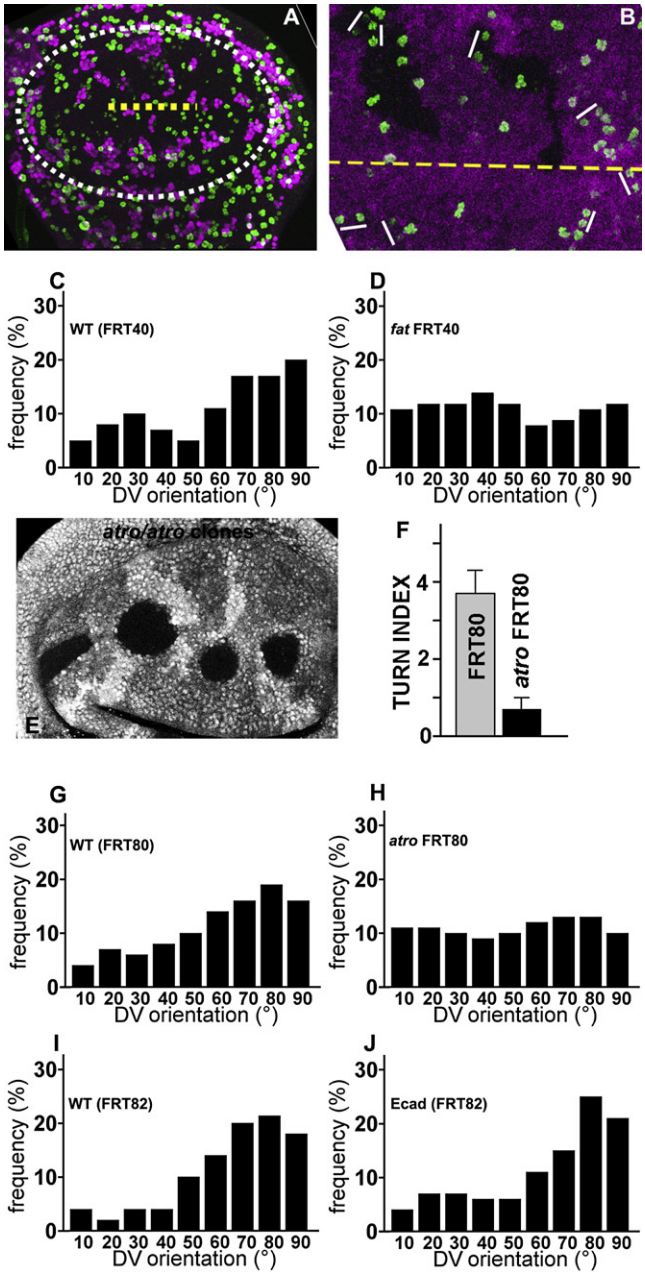

Figure 3. Role of Planar Polarity Genes in Mitotic Orientation

(A) Wing imaginal disc labeled for mitotic figures (green) and random clones labeled for β-galactosidase expression (magenta). The wing pouch region is outlined by the dotted white circle. Within the wing pouch, wild-type clones in a wild-type background tend to orient toward the distal dorsoventral boundary (dotted yellow line). Outside the wing pouch, clones tend to orient circumferentially [8].

(B) Higher magnification of a central wing pouch, showing wild-type clones in a wild-type background marked by absence of β-galactosidase labeling (magenta). Orientation of anaphase and telophase cells is indicated by white lines. Position of the dorsoventral boundary (dashed yellow line) was determined by labeling for the Senseless protein (not shown).

(C) Within clones of wild-type cells (*FRT40*) in a wild-type background (*FRT40/+*), mitoses in the wing pouch more often orient perpendicular to the dorsoventral boundary, as reported previously [8]. n = 100.

(D) Within clones of *fat* mutant cells (*ft[NY1] FRT40*), mitoses in the wing pouch show no orientation with respect to the dorsoventral boundary. n = 66.

(E) Clones of *atro* mutant cells (*atro[35] FRT80*), marked by the absence of β-galactosidase staining, are similar in size to control clones but are round with smooth boundaries. Reciprocally recombinant wild-type clones express two doses of the β-galactosidase transgene.

(F) The boundary turn index of *atro* mutant clones is significantly lower than that of control clones. n = 10 and 12, respectively. Error bars represent SEM.

(G) Within clones of wild-type cells (*FRT80*), mitoses in the wing pouch tend to orient toward the dorsoventral boundary. n = 243.

(H) Within clones of *atro* mutant cells (*atro[35] FRT40*), mitoses in the wing pouch show no orientation with respect to the dorsoventral boundary. n = 232.

(I) Within clones of wild-type cells (*FRT82*), mitoses in the wing pouch tend to orient toward the dorsoventral boundary. n = 49.

(J) Within clones of cells overexpressing E-cadherin (mosaic analysis with a repressible cell marker [MARCM] experiment in the *FRT82* strain background; see Experimental Procedures for details), mitoses in the wing pouch continue to orient toward the dorsoventral boundary. n = 54.

development to replace dead cells requires cell rearrangement to reconstitute organ shape in addition to cell number regulation, and that in imaginal discs this occurs by mitotic reorientation. Because each organ is the sum of its constituent clones of cells, behavior that affects the shapes of clones ultimately defines the shape of each organ. The clone shapes reveal the ancestry of individual cells over the course of development, and irregular boundaries reflect the preferential origin of cells from one cell population.

Reorientation in response to cell death requires the *fat/dachsous* PCP pathway. This pathway also affects the cell division axis in normal wing development, although very little cell death occurs in normal development [20, 24]. Developmental signals and cell-death-derived signals must converge on mitotic orientation at some level upstream of PCP genes. It is not known at present how cells sense nearby apoptosis. Possible mechanisms include alterations in Fat or Dachsous activity levels in dying cells, which would have nonautonomous effects [12]; expression of growth factors by dying cells [9]; cell polarization in the course of engulfing dead cell fragments [25]; or mechanical stretching of the epithelium when cell numbers are reduced [26]. PCP proteins could sense one or more such signals and define the polarized cellular response; alternatively, PCP genes might be necessary to implement a polarity that is sensed by other proteins.

The mitotic orientation role of PCP genes in development may be conserved: the mammalian *Fat-4* gene is required for oriented cell division in kidney tubule elongation [27], and polarized migration of smooth muscle cells following arterial damage or atherosclerosis depends on mammalian Fat-1 and Atrophins [28, 29]. It will be interesting to determine whether PCP genes are involved in the response to damage and cell death in mammals, and whether defects in such processes contribute to conditions such as dentatorubral-pallidoluysian atrophy. It seems plausible that cell migration or convergent extension could mediate regulation in response to damage also, in tissues where such processes are more prominent [30–32].

Experimental Procedures

Mosaics

Mosaic clones were obtained via the FRT-FLP method for mitotic recombination [33]. Flies were maintained at 25°C. Heat shock was usually performed at 37°C for 1 hr. Minute genotypes were generally heat shocked 84 ± 12 hr after egg laying and dissected 72 hr later, unless indicated otherwise. Other genotypes were heat shocked at 60 ± 12 hr after egg laying and dissected 60 hr later. Except for in Figure 1H, the *M/+* genotypes shown in Figure 1 and Figures 4A and 4C were heat shocked for 30 min at 108 ± 12 hr after egg laying and dissected 48 hr later.

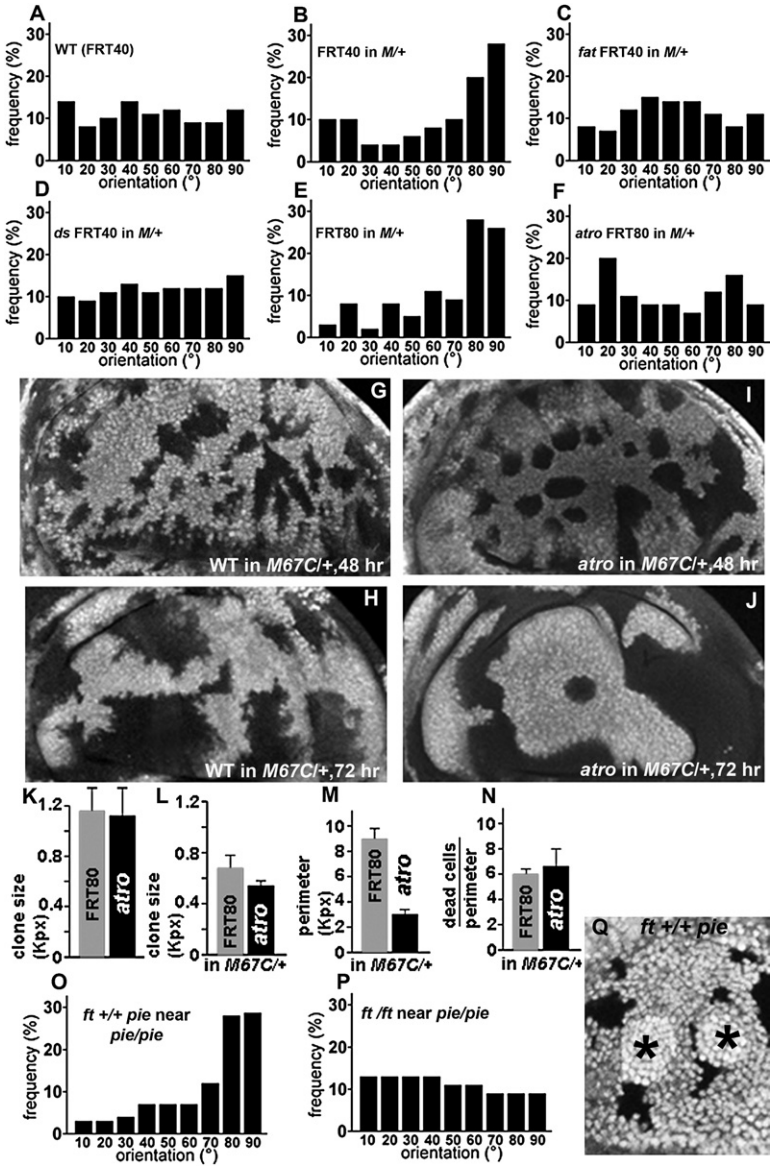

Figure 4. Mitotic Orientation and Cell Death

(A) Within clones of wild-type cells (*FRT40*), mitoses in the wing pouch show little orientation toward the clone boundaries. n = 118.

(B) Within clones of wild-type cells (*FRT40*), mitoses tend to orient toward nearby boundaries with *M(2)21C/+* regions. n = 50.

(C) Within clones of *fat* mutant cells (*ft[NY1] FRT40*), mitoses in the wing pouch show little orientation toward nearby boundaries with *M(2)21C/+* regions. n = 136.

(D) Within clones of *ds* mutant cells (*ds FRT40*), mitoses in the wing pouch show little orientation toward nearby boundaries with *M(2)21C/+* regions. n = 105.

(E) Within clones of wild-type cells (*FRT80*), mitoses in the wing pouch tend to orient toward the clone boundaries. n = 159.

(F) Within clones of *atro* mutant cells (*atro[35] FRT80*), mitoses in the wing pouch show little orientation toward nearby boundaries with *M(3)67C/+* regions. n = 92.

(G and H) Wild-type clones (lacking β-galactosidase labeling) in the *M(3)67C+* background 48 hr (G) and 72 hr (H) after induction.

(I and J) Clones homozygous for *atro* (lacking β-galactosidase labeling) in the *M(3)67C+* background 48 hr (I) and 72 hr (J) after induction. Despite their smooth boundaries, *atro* clones show growth comparable to controls in the *M/+* background.

(K) Quantification of wild-type (FRT80) and *atro* mutant clone growth in a wild-type background. Clones were measured 72 hr after induction.

(L) Quantification of wild-type (FRT80) and *atro* mutant clone growth in an *M/+* background. The *atro* clones were smaller on average, but the difference was not statistically significant (t test, p = 0.14). Clones were measured 48 hr after induction.

(M) *atro* clones (*M/+* background) have shorter perimeters than FRT80 clones, as a consequence of their round shape and smooth boundaries.

(N) Apoptotic *M/+* cells are seen at boundaries in *atro* and wild-type clones at comparable rates when normalized to boundary length. Error bars in (K)–(N) represent SEM.

(O) Within regions of control cells ($pie^{E1\text{-}16}/ft^{NY1}$), mitoses tend to orient toward nearby boundaries with *pie* homozygous clones. n = 69 mitoses analyzed from 54 clones.

(P) Within ft^{NY1} homozygous clones, mitoses tend to orient toward nearby boundaries with *pie* homozygous clones. n = 88 mitoses analyzed from 71 clones.

(Q) ft^{NY1} *ubi-GFP*/$pie^{E1\text{-}16}$ wing disc containing clones homozygous for $pie^{E1\text{-}16}$ (lacking labeling for GFP) adjacent to clones homozygous for ft^{NY1} (asterisks indicate two doses of GFP transgene). Note that *pie* clone boundaries are irregular, except for where they border *ft* clones and become smooth.

Turn Index

Because the difference in clone shapes was not captured well by the circularity statistic (area/perimeter), we used a zero-crossing statistic that we called the "turn index" to describe the irregularity of clone boundaries. The turn index was defined as the number of turns greater than 90° leftward encountered during a clockwise circuit of the clone perimeter, normalized against the length of the clone perimeter (see Figure 1G).

Mitotic Orientation

Most imaginal disc cells divide symmetrically in the plane of the epithelium. The axis of division was measured in anaphase and telophase cells labeled by antibodies for phosphohistone H3, a marker for mitotic chromatin. We found this superior to spindle components such as γ-tubulin because of both the extensive tubulin labeling of these epithelia and the need to define cell-cycle stage given the potential for the spindle to rotate early in mitosis. Accuracy in distinguishing anaphase and telophase nuclei from pairs of nearby cells in prophase was validated by labeling some samples for the cortical protein Merlin to outline cell boundaries. The dorsoventral boundary was positioned by using antibodies against the Senseless protein, which is expressed in neurons along the future wing margin [34]. To quantify orientation with respect to clone boundaries, we defined the boundary angle as follows: Projecting the division axis toward the Minute cell population, the boundary was defined as the straight line connecting two points approximately one spindle length apart in each direction from the intersection, along the interface between the Minute and non-Minute populations, as shown in Figure 2B. Following this procedure provided objectivity in cases where the boundary between cell populations was irregular. Only mitotic figures close to the clone boundary were assessed, because mitoses in the clone interior may be equidistant to boundaries in several directions.

Genetic Strains and Immunohistochemistry

See Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and one figure and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01702-3.

Acknowledgments

We thank H. McNeill, P. Meier, J.P. Vincent, and the Bloomington Drosophila Stock Center for *Drosophila* strains and R. Fehon and the Developmental Studies Hybridoma Bank for antibodies. The manuscript was improved by comments from A. Jenny, L. Johnston, H. McNeill, N. Sibinga, and D. Tyler. Data in this paper are from a thesis to be submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the Graduate Division of Biomedical Sciences, Albert Einstein College of Medicine, Yeshiva University. This work was supported by National Institutes of Health grant GM61230.

Received: September 10, 2008
Revised: August 24, 2009
Accepted: September 3, 2009
Published online: October 22, 2009

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